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The osteoblast in regulation of tumor cell dormancy and bone metastasis

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HIGHLIGHTS

- Disseminated tumor cells (DTCs) are often located in close proximity to osteoblasts.
- Osteoblasts maintain cancer cell dormancy through secreted factors and direct cell to cell contacts.
- Micrometastases are surrounded by osteoblast lineage cells that support cancer growth via various mechanisms.
- Different osteoblast subpopulations might have distinct functions in bone metastases.
- Increasing osteoblast activity in osteolytic metastases might serve as a strategy to treat the disease. However, more research is needed to better understand the complex role of osteoblasts in disease progression.

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ABSTRACT

Breast and prostate cancer are among the most common malignancies worldwide. After treatment of the primary tumor, distant metastases often occur after a long disease-free interval. Bone is a major site for breast and prostate cancer metastasis and approximately 70% of patients with advanced disese suffer from osteolytic or osteoblastic bone metastases, a stage at which the disease is incurable. In bone, the disseminated tumor cells (DTCs) can become quiescent or "dormant", a state where they are alive but not actively dividing. Alternatively, the cancer cells can proliferate, disturb the bone homeostasis, and form metastatic lesions. The fate of cancer cells is largely dependent on the bone microenvironment, particularly the bone forming osteoblasts and bone resorbing osteoclasts. Osteoblasts originate from mesenchymal precursors through a tightly regulated cascade. The main function of osteoblasts is to synthesize bone matrix, coordinate mineralization and maintain bone remodeling by regulating osteoclast activity and bone resorption. In metastatic bone environment, osteoblasts can create a niche within the bone where DTCs cells become dormant and induce quiescence in cancer cells keeping them in a non-proliferative state. Osteoblasts also contribute to metastatic outgrowth and actively promote tumor growth in bone. In this article, we review the recent literature on the role of osteoblasts in cancer cell dormancy and bone metastasis and describe the underlying mechanisms by which osteoblasts regulate cancer cell fate in bone. In addition, we discuss the possibility of targeting osteoblasts to treat osteolytic bone metastases.

1. Introduction

Bone metastasis is a debilitating disease associated with pathological fractures, severe pain, and reduced quality of life. Bone metastases are frequent complications of many cancers. In breast and prostate cancer, bone represents the most common metastatic site and approximately 70 % of patients with advanced cancer have bone involvement [1]. The most frequent skeletal sites of metastases are the spine, pelvis, ribs, proximal femur and the skull. Bone metastases often lead to skeletal related events (SREs) that include fractures, spinal cord compression, bone pain and disability, contributing to morbidity and mortality of patients.

The multistep process of metastasis starts from the primary tumor. In the primary site, cancer cells undergo epithelial-mesenchymal transition (EMT), intravasate to enter the circulation, migrate in the blood stream as circulating tumor cells (CTCs) and disseminate to distant organs. In

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the distant site, disseminated tumor cells (DTCs) have to adapt to their new microenvironment and either remain quiescent or proliferate to form metastases. DTCs are frequently detected in the bone marrow of patients already at the time of diagnosis and after tumor resection [2]. Although presence of DTCs in the bone marrow is predictive of future relapse, metastatic relapse can occur years or even decades after successful removal of the primary tumor. This indicates that metastasis is an early process but DTCs can enter a dormant state in the distal organ. Dormant DTCs survive adjuvant therapies and finally outgrow once the environmental conditions in the bone are more permissive [3].

Bone is a metabolically active organ that is constantly remodeled throughout life. During bone remodeling, old bone is removed by bone-resorbing osteoclasts and new bone is formed by bone-forming osteoblasts [4]. In physiological conditions, bone resorption and bone formation are tightly balanced and bone mass remains constant. Several diseases, including bone metastases, disturb bone remodeling leading to pathological bone loss or gain. In breast cancer bone metastases, cancer cells secrete cytokines including parathyroid hormone-related peptide (PTHrP) that stimulate the osteoblasts to produce excessive amount of receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL activates the osteoclasts to differentiate and resorb the bone matrix [5]. Upon resorption, matrix-bound growth factors such as transforming growth factor β1 (TGF-β1) are released and further support tumor growth. This so called "vicious cycle of bone metastases" perpetuates tumor growth and bone destruction eventually leading to osteolytic lesions. Prostate cancer bone metastases often cause osteosclerotic lesions due to a pathologically increased osteoblast activity and formation of woven bone [6].

Osteoblasts are cuboidal cells residing on the bone surfaces [7]. The main function of osteoblasts is to synthesize bone matrix and coordinate mineralization. The organic bone matrix is composed of crosslinked collagen and specialized proteins such as osteocalcin and osteopontin. To mineralize the matrix, osteoblasts produce hydroxyapatite that is deposited into the newly synthesized organic matrix. Besides forming bone, osteoblasts are implicated in global energy balance by secreting endocrine factors, including the metabolic hormones osteocalcin and lipocalin [8]. In addition, osteoblasts are crucial for the maintenance of bone homeostasis by regulating bone resorption. They secrete RANKL to activate the osteoclasts and osteoprotegerin (OPG), a decoy receptor of RANKL to halt osteoclast differentiation and bone resorption [9]. Osteoblasts are relatively short living cells. Once bone formation is completed, osteoblasts can undergo apoptosis, become inactive lining cells on the bone surfaces or become embedded in the bone matrix as terminally differentiated osteocytes [4].

Osteoblasts arise from cells of mesenchymal origin. The fundamental discovery of multipotent, plastic adherent cells that have the potential to self-renew and differentiate into various mesenchymal lineages including osteoblasts, chondrocytes and adipocytes occurred already in the 1960s [10]. These cells were termed as mesenchymal stem/stromal cells (MSCs) [11]. Recent studies have shown that MSCs in fact consist of a heterogenous population of cells with different capabilities and characteristics. A subset of MSCs termed "skeletal stem cells (SSCs)" represent cells that reside in the bone marrow, periosteum and growth plate and have the capacity to differentiate into osteoblasts, chondrocytes and adipocytes. Consistent with the compartment-specific location of the stem cells, osteoblasts can arise from multiple origins including growth plate chondrocytes, bone marrow stromal cells and quiescent bone lining cells in response to regenerative and anabolic stimuli [7]. Recent reports have shown that osteoblasts derived from different cellular sources have different functions [12]. New technologies such as lineage tracing and single cell sequencing have facilitated the identification of different (sub)populations. For instance, osteoblast molecular and functional heterogeneity has been analyzed by single cell sequencing using *Col1a1-Cre;R26R-Lyn-Venus*-derived calvaria osteoblasts. These studies showed that osteoblasts can be distinguished based on their differentiation status and lineage marker genes as well as their function as determined by gene ontology analysis [13]. However, the role of different populations in metastatic bone diseases is largely unknown. Since the concept of osteoblast heterogeneity is emerging and a standard nomenclature for distinct osteoblast subpopulations and their functions is not fully established, in this manuscript we specify the state and origin of osteoblasts whenever possible. When not further specified, we use the term "osteoblast", acknowledging that an osteoblast is not "just" an osteoblast but a heterogenous group of osteoblast-lineage cells.

In this article, we review the current literature on the role of osteoblasts in early stages of bone metastases and cancer cell dormancy. In addition, we describe how osteoblasts contribute to cancer relapse and metastatic growth in bone and discuss the role of osteoblasts as cellular target to treat bone metastases.

2. Osteoblasts regulating cancer cell homing to bone

Organ trophism of metastatic cancers is regulated by cancer cell-intrinsic factors and by the local microenvironment. Bone is a unique environment in terms of its mineral content, matrix composition, rigidity, and high calcium concentration [14]. Furthermore, bone is hypoxic environment with acidic pH [15]. In addition to the physiochemical properties, several secreted molecules attract cancer cells to bone. These include matrix embedded proteins but also molecules secreted by the osteoblasts. RANKL is secreted by osteoblasts and high expression of RANK in hormone receptor negative primary breast tumors is associated with poor relapse-free survival and high risk of bone metastases, indicating a role for RANKL-RANK-axis in bone metastases [16,17]. Indeed, in vitro, RANKL promotes migration of RANK expressing breast and prostate cancer cells suggesting that RANKL might attract cancer cells to bone [18,19]. However, inhibition of RANKL by its decoy receptor OPG did not reduce the number of cancer cells disseminated to bone in a mouse model of breast cancer bone metastasis [20]. Consistently, adjuvant treatment with Denosumab, a monoclonal antibody against RANKL had no effect on disease recurrence in patients with early-stage breast cancer and only modestly increased bone metastasis-free survival in patients with non-metastatic castration-resistant prostate cancer [21,22]. Together the pre-clinical and clinical data suggest that osteoblast-derived RANKL does not play a major role in cancer cell homing to bone. Instead, periostin, which is produced by osteoblasts and other stromal cells has been shown to support cancer cell adhesion by binding to a_vb_3 integrin [23]. In addition, changes in osteoblast function also alter cancer cell homing to bone. Senescent osteoblasts secrete Interleukin 6 (IL-6) that activates osteoclasts and bone resorption. Increased bone resorption in turn increases breast cancer cell seeding in bone and supports metastasis formation [24].

3. Osteoblasts in cancer cell dormancy

Once in the bone, DTCs can enter a dormant state and stay quiescent for years. Although the presence of DTCs predicts bone relapse, not all patients with DTCs develop bone metastases, suggesting that the bone marrow microenvironment affects DTC fate [25,26]. Several studies have shown that upon arrival to the bone, single DTCs are in close contact with the bone marrow vasculature and osteoblast lineage cells (MSCs, osteoprogenitors, pre-osteoblasts and osteoblasts) but not osteoclasts [27–29] suggesting that endothelial and/or osteoblast lineage cells support cancer cell dormancy (Fig. 1). Indeed, the vascular niche has been shown to maintain tumor cell dormancy through several mechanisms including thrombospondin-1 (TSP-1) and the CXCR-4/CXCL-12 axis [30,31]. More recently, also the endosteal niche, in particular the osteoblasts and their precursors have been implicated in dormancy. In vitro, human MSC-derived osteoblasts were shown to induce a quiescent phenotype of 3384T breast cancer cells demonstrated by growth retardation and reduced expression of proliferation markers [32]. Consistently, in a 3D model in which MC3T3-E1 osteoblasts were cultured in a bioreactor to form mineralized tissue, a bone-like

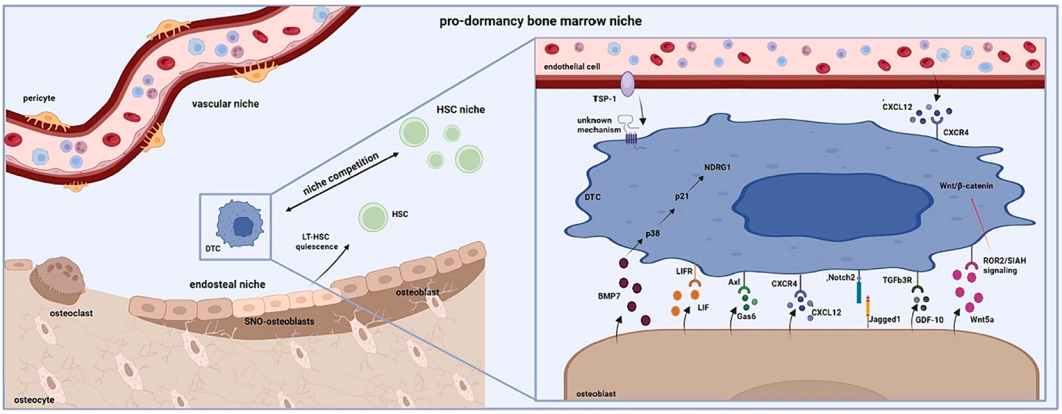

Fig. 1. Regulation of the disseminated tumor cell pro-dormant milieu by osteogenic cells. Disseminated tumor cells (DTCs) have been found in the proximity of the perivascular niche, a microenvironment associated with the expression of pro-dormancy factors thrombospondin-1 (TSP-1) and CXCL12. Additionally, dormant DTCs localize in osteoblast rich areas in the bone, the endosteal niche. DTCs compete with hematopoietic stem cells (HSC) and induce HSC re-location within the bone microenvironment. A spindle-shaped N-cadherin+/CD45- subpopulation of osteoblasts (SNO-cells) induce long-term hematopoietic stem cells (LT-HSC) quiescence via Jagged1, a similar mechanism that is considered to maintain tumor cell dormancy by osteoblasts. Dormancy is induced by osteoblast derived factors GDF-10 and Gas6 which act through reduced expression of TNF-α, IL-1β and IL-6, in addition to Leukemia inhibitory factor (LIF)-LIF receptor (LIFR)-STAT3 signaling axis. Additionally, increased expression of chemokines CXCL-12, TSP-1 and tropomyosin secreted by osteoblasts maintains a dormant state. Osteoblast derived ligand Wnt5a activates ROR2/SIAH2 signaling to suppress Wnt/β-catenin signaling promoting dormancy. Bone morphogenic protein 7 (BMP7), secreted by osteoblasts, activates p21/p38/MAPK signaling and thus further upregulates N-myc downstream regulated gene 1 (NDRG1) expression to suppress metastasis. Created with BioRender.com.

environment inhibited the growth of non-metastatic MDA-MB-231-BRMS1 variant [33]. Since the BRMS1 readily proliferated in a 2D model, the findings suggest that osteoblasts induce a growth arrest of these cells. Similarly, MCF-7 cells entered a dormant stage when co-cultured with human or mouse osteoblasts in a 3D model [34]. Stimulation with cytokines related to bone repair and remodeling, particularly tumor necrosis factor α (TNF-α) and IL-1β released the dormancy phenotype and stimulated cancer cell proliferation. The escape from dormancy was associated with increased production of prostaglandin 2 (PGE2). Exogenous PGE2 stimulated cancer cell proliferation while inhibition of PGE with COX inhibitor indomethacin or by PGE2 receptor antagonist reversed the cytokine-induced effect on proliferation, suggesting that PGE2 is a key determinant between dormancy and proliferation in the bone microenvironment [34].

3.1. *Osteoblast subpopulations in cancer cell dormancy*

Further characterization of the metastatic bone niche has revealed that disseminated cancer cells "educate" a subpopulation of osteoblasts to support their dormancy state [35]. These "educated" osteoblasts were shown to express Runx2, osteocalcin and osteopontin. They also displayed increased expression of collagen type 1 (Col1) and matrix metalloproteinase 3 (MMP3) that activated the cell cycle inhibitor p21 in triple-negative breast cancer cells leading to suppressed proliferation. In contrast, "uneducated" osteoblasts were distinguished based on increased IL-6, alpha smooth muscle actin (αSMA) and vascular endothelial growth factor (VEGF) expression, while showing no difference in Col1, alkaline phosphatase (ALP) and MMP3. These data suggest that cancer cells "educate" the osteogenic microenvironment to adapt towards their benefit of a pro-dormant or pro-proliferative milieu [35]. These findings also suggest that different osteoblast subpopulations might have distinct roles in regulating dormancy.

Undifferentiated, spindle shaped N-cadherin+/CD45- osteoblasts (SNO) is a small, specific cell population located on the endosteal surfaces. SNO cells have been shown to maintain quiescence of long-term hematopoietic stem cells (LT-HSCs) through cell–cell contact and paracrine factors. Since LT-HSCs localize in the same niche with DTCs [36], the question arises whether SNOs also maintain DTC quiescence. Indeed, a competition assay revealed that the presence of human-derived MDA-MB-231 or mouse-derived 4T1 breast cancer cells adjacent to the endosteal surfaces reduced the engraftment of HSCs, suggesting that DTCs and HSCs compete for niche occupancy [37]. Interestingly, DTCs that did not cause relapse (dormant DTCs) preferentially located on surfaces that were enriched in SNOs. These N-cadherin+ SNOs are less differentiated than non-SNO osteoblasts and characterized by high expression of Jagged-1, a Notch ligand implicated in LT-HSC quiescence. Similar to LT-HSCs, SNOs reduced proliferation of triple-negative breast cancer cells (MDA-MB-231 and 4T1) in vitro through Jagged-1-Notch signaling. Interestingly, no growth retardation was observed in ER+ MCF-7 cells that do not express Notch2 receptor, suggesting that SNO-mediated dormancy functions through Notch2 in aggressive breast cancer cells [37]. Accordingly, MDA-MD-231 cells expressing low levels of Notch2 resulted in rapid progression to overt metastases in vivo, while cells with high expression of Notch2 proliferated significantly slower. Finally, disturbing the interaction between SNOs and DTCs in vivo using γ-secretase inhibitor DBZ mobilized DTCs from the endosteal surfaces. However, no overt metastases and osteolytic lesions were observed, suggesting that other mechanisms contribute to dormant DTC activation. Indeed, MDA-MB-231 cells exhibiting high Notch2 expression have also high expression of N-Cadherin that facilitates their attachment to SNOs and contributes to proliferation in vitro [38]. Whether the mechanism plays a role in vivo remains to be elucidated.

3.2. *Osteoblast-derived secreted factors regulating dormancy*

Osteoblast-derived inflammatory cytokines play important roles in maintaining the balance between cancer cell dormancy and growth. Leukemia inhibitory factor (LIF) belongs to the IL-6 family and promotes breast cancer cell dormancy in the bone [39]. Binding of LIF to the LIF receptor (LIFR) on cancer cells induces the expression of TSP-1, tropomyosin and other dormancy promoting genes while inhibitor of LIFR in MCF-7 cells reduced p53 expression [39]. These findings indicate that LIF-LIFR signaling is important in the maintenance of breast cancer cell dormancy in bone. Similarly, osteoblast-derived chemokines CXCL-12 and growth-arrest-specific gene-6 (Gas6) promote breast and prostate cancer cell dormancy through CXCR4 signaling and inhibition of pro-inflammatory factors TNF-α, IL-1β and IL-6, respectively [40,41].

In addition to Gas6 signaling, osteoblasts contribute to prostate cancer dormancy through various mechanisms. Osteoblast-derived bone

morphogenetic protein 7 (BMP7) induces dormancy in PC-3 cells activating p38 and p21, leading to an increased expression of a metastasis suppressor gene N-myc downstream-regulated gene 1 (NDRG1). Withdrawal of BMP7 abrogated the suppressive effect of osteoblasts in vivo promoting metastatic outgrowth [42]. Besides BMP signaling, Wnt signaling in the osteogenic niche regulates prostate cancer cell dormancy. A non-canonical Wnt ligand Wnt5a activated the receptor tyrosine kinase-like orphan receptor 2 (Ror2)/SIAH signaling axes resulting in inhibition of the canonical Wnt/β-catenin pathway and subsequently promoting dormancy [43]. Silencing Wnt5a restored the growth ability of prostate cancer cells.

4. Osteoblasts regulating cancer cell colonization and growth

One of the relatively unanswered questions is what triggers the reawakening of dormant tumor cells in the bone. While increased osteoclast activity and bone resorption has been shown to activate dormant cancer cells, also the osteoblasts have an important role in the process. For instance, metastatic breast cancer cell derived Jagged1 has been shown to activate Notch signaling in osteoblasts [44]. Consequently, osteoblasts secrete increasing amount of IL-6 and connective tissue growth factor (CTGF) leading to tumor growth. Furthermore, tumor-derived Jagged1 activates osteoclast differentiation and bone resorption leading to the release of TGF-β and progression of the vicious cycle [44]. Consistently, a fully human monoclonal antibody against Jagged1, 15D11, showed efficacy in preventing bone metastasis. Interestingly, the chemotherapeutics Paclitaxel and Cisplatin induce the expression of Jagged1 in osteoblasts and MSCs which resulted in a crosstalk to the cancer cells, activated Notch signaling and chemoresistance. The combination of 15D11 and chemotherapy leads to sensitization of bone metastatic cancer cells towards chemotherapy by reducing spontaneous bone metastasis [45].

4.1. Cell-cell interactions mediating cancer cell colonization and growth

In addition to the indirect effect on tumor growth through osteoclasts, osteoblasts directly support cancer cell colonization and growth. Similar to single DTCs, micrometastases frequently reside in the osteogenic niche. In fact, breast cancer micrometastases have been shown to be surrounded by ALP (ALP+) and Collagen 1 (Col1+) -positive osteogenic cells both in immunocompromised (MCF-7, MDA-MB-361) and immunocompetent (4T1, 4TO7) models of bone metastases [29]. Quantitative analysis of the niche cells showed that around 80 % of the surrounding cells were ALP+, 40–65 % Col-I+ but only 20 % were positive for the osteoclast marker cathepsin K (CTSK). Interestingly, in tumor free metaphysis only 10 % were ALP+ and 3 % Col-I+ indicating an increase of ALP+ and Col-I+ cells in the bone upon cancer cell arrival. While the niche cells were also expressing Nestin, CD31, αSMA and CD45, the frequency of osteogenic cells was more pronounced. Furthermore, ALP+ and Col-I+ cells also expressed the osteoblast transcription factors Osterix and Runx2 as well as β-catenin and LEF-1 indicating active osteogenesis. This was confirmed by an increased osteoid formation on bone adjacent to microlesions in bone metastasis [29]. Supporting these findings, reduced osteoblast activity and bone formation was associated with impaired bone metastasis incidence and growth in an intracardial model of bone metastases [28]. In this study, deletion of a transcriptional regulator Tgif1 in osteoblasts reduced the migration of breast cancer cells in vitro and tumor growth in vivo. RNA Seq and SILAC analysis revealed that osteoblasts lacking Tgif1 abundantly express semaphorin3E, an inhibitor of cell migration providing a possible mechanism by which osteoblasts mediate cancer cell growth [28].

The direct connection between cancer cells and osteoblasts suggests that the cells communicate, at least in part, via gap junctions or cell surface proteins (Fig. 2A). Organoid 3D cell culture models have shown that the crosstalk between breast cancer cells (MCF-7, MDA-361, 4T1, 4TO7) and MSCs (mouse and human MSCs and FOB1.19 cells) accelerates tumor growth compared to organoids lacking MSCs [29]. However, these effects could not be seen using RAW 264.7, monocytes or differentiated osteoclasts. This crosstalk was maintained with tight heterotypic adherens junctions (hAJ) between E-cadherin expressed by cancer cells and N-cadherin in ALP+ cells leading to an upregulation of mammalian target of rapamycin (mTOR) signaling in early bone colonization [29]. The inhibition of the hAJ with neutralizing antibodies resulted in smaller bone lesions and reduced proliferation in an in vivo model using MCF7 breast cancer cells. Additionally, treatment with an mTOR inhibitor Torin1 delayed cancer colonization to bone at early

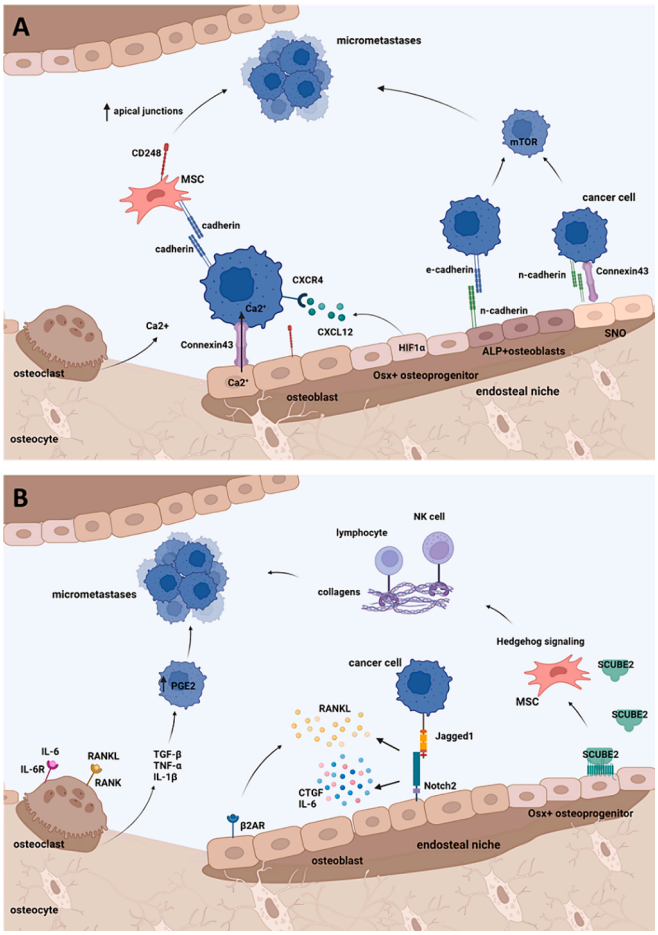

Fig. 2. Osteoblast lineage cells drive cancer cell colonization and proliferation in the metastatic niche. Triggered by microenvironmental cues, DTCs proliferate and colonize the bone. **(A)** Hypoxia in the bone marrow environment upregulates hypoxia-inducible factor α (HIF1α) by Osx+ osteoprogenitors and increases the CXCL12 expression and dissemination. The hypoxic bone microenvironment also increases osteoclasts and bone resorption releasing matrix embedded factors. These include Ca2+ that is absorbed by osteoblasts. Tumor cells are connected to osteoblasts via the gap junction connexin 43 that facilitates the uptake of Ca2+ from the osteoblasts to promote cancer cell proliferation. Direct interactions between cancer cells and osteoblast lineage cells via Connexin 43, E-cadherin and N-cadherin activate DTC proliferation by stimulating e.g. mTOR signaling. Upregulation of CD248 is associated with an increase in apical junctions and cancer cell invasion. **(B)** The matrix released TGF-β activates the Notch ligand Jagged1. Jagged1/Notch signaling stimulates the secretion of osteoclast-promoting factors RANKL, CTGF and IL-6. The stimulation of β2AR in osteoblasts leads to the release RANKL. Also, the cytokines TGF-β, TNF-α and IL-1β are pro-proliferative signals through increased production of PGE2. SCUBE2 increases osteoblast differentiation via Hedgehog signaling in MSCs. Osteoblasts deposit collagen which suppresses the immune response of NK-cells and other lymphocytes against the tumor cells. Created with BioRender.com.

stages of metastasis, but not at later stages of tumor proliferation [29].

The cancer cells also benefit from the high calcium levels in the bone during early stages of colonization. The intrinsic intracellular calcium concentration [Ca^{2+}] in cancer cells is relatively low, indicating that a full absorption is not occurring. However, the co-localization with osteoblasts increased the Ca^{2+} level in cancer cells via gap junctions. This resulted in depleted calcium levels in osteoblasts and consequently promoted cancer progression [14]. Consistently, interruption of cancer cells' calcium uptake leads to a reduction in bone colonization of MCF7 breast cancer cells. In addition, treatment with the gap junction and calcium signaling inhibitor arsenic trioxide (AS_2O_3) was shown to disturb the calcium signaling and suppressed bone metastasis without causing side effects [14].

Recently, the cell surface protein SCUBE2 was identified to be highly expressed in ER-positive luminal breast cancer bone metastasis and showed a positive correlation with bone metastasis risk [46]. Single cell RNA (scRNA)-sequencing identified 24 clusters of $CD45^-$ stromal cells in the metastatic niche in BALB/c nude mice injected with MCF7 breast cancer cells with and without SCUBE2 knockdown (Fig. 2B). Interestingly, SCUBE2 knockdown in MCF7 cells reduced the number of osteolineage cells in the cluster of ALP+ osteoblasts and showed a downregulation of osteolineage genes including ALP, osteocalcin (Bglap) and osterix (Sp7), as well as pathways involving ossification and collagen formation. Accordingly, immunohistochemical and immunofluorescent analysis on SCUBE2 overexpression showed an osteoblast enrichment in the early metastatic niche while a reduction of ALP+ osteoprogenitors was observed in SCUBE2-deficient tumor cells. The proposed mechanism involved SCUBE2-mediated activation of Hedgehog signaling in MSCs via release of tumor-derived SHH leading to pre-osteoblast differentiation. This further led to an augmented deposition of collagens by differentiated osteoblasts in the bone metastatic niche. Osteoblast-derived collagen mediated the NK immune cell suppression by binding to the inhibitory collagen and leukocyte-specific receptor LAIR1, indicating that osteoblasts can mediate the immune response in the tumor microenvironment. Remarkably, breast cancer bone metastasis was inhibited after targeting Hedgehog signaling with the administration of Sonidegib or with LTMA16D5, an SCUBE2 neutralizing agent [46].

4.2. Hypoxia signaling as a regulator of colonization and growth

Besides being a reservoir for proteins, minerals and enzymes, the bone is also special in terms of pH and oxygen supply. Bone is a hypoxic environment, and the hypoxic conditions regulate bone homeostasis [47]. In addition, hypoxia-inducible factors (HIFs) and HIF signaling are associated with tumor growth, angiogenesis and EMT [48]. HIF1α has been shown to be upregulated in a hypoxic primary breast cancer microenvironment [49]. Deletion of HIF1α in the mammary of a PyMT-driven spontaneous breast carcinoma mouse model reduced bone metastatic burden while lung metastasis was increased [50]. Consistently, HIF2α-deleted tumors exhibited a decreased bone dissemination, while the activation of HIF-pathways using a mammary-specific deletion of von Hippel Lindau (Vhl) increased tumor dissemination to bone. Neither HIF2α nor Vhl-deletion had an effect on lung dissemination. While bone mass was unchanged upon HIF2α or Vhl-deletion, mice with HIF1α-deficient tumors had reduced bone mass, suggesting that disruption in bone homeostasis might promote lung colonization [50]. In addition to the primary tumor, activated HIF signaling in osteoprogenitors has been shown to mediate breast cancer cell dissemination to the bone (Fig. 2B). Osteoprogenitors are located in hypoxic bone niches and activated HIF signaling in Osx-positive cells increased CXCL12 levels in the blood [51]. Specifically, HIF1α and VHL, influence tumor cell colonization to the bone. While the ablation of HIF signaling in osteoblasts ($\Delta HIF1\alpha^{OSX}$ mice) resulted in reduced tumor cell dissemination, inactivation of von Hippel Lindau ($\Delta Vhlh^{OSX}$ mice) increased tumor colonization [51]. Furthermore, activated HIF signaling in Osx-expressing osteoprogenitors promoted systemic breast cancer growth and dissemination to the lung. Together these studies highlight the importance of HIF signaling in breast cancer dissemination in bone and other organs in a site-specific and systemic manner.

4.3. Osteoblast-dependent interactions within the bone microenvironment

Osteoblasts are also implicated in stress-induced breast cancer bone metastases. Chronic emotional stress is associated with breast cancer recurrence, reduced survival, and poor prognosis [52]. Chronic stress stimulates sympathetic nervous system, causing the release of peripheral catecholamines that stimulate post-synaptic β-adrenergic receptors (βARs). Osteoblasts express β2AR and stimulation of osteoblasts with βAR agonists increases RANKL expression (Fig. 2B). Consistently, inhibition of RANK in MDA-MB-231 breast cancer cells prevented the increased bone metastases that occurs upon pharmacologic or endogenous activation of sympathetic nervous system [53]. Besides secreting RANKL, osteoblasts respond to isoproterenol-induced β2AR activation by increasing the expression and secretion of VEGF-a [54]. This led to an increased vessel formation in an in vitro angiogenic assays. Interestingly, deletion of β2AR in germ line or in Col1-expressing osteoblasts restored the isoproterenol-induced increase in VEGF-expressing osteoblasts and vessel density in bone. Furthermore, both genetic inhibition of β2AR in Col1-expressing osteoblasts and pharmacologic inhibition of VEGF - VEGF receptor interaction reduced the increased incidence of bone metastases and bone lesions induced by isoproterenol [54]. Further in vitro experiments revealed that isoproterenol treatment increased the abundance pro-inflmammatory cytokines IL-1β and IL-6 in differentiated MSCs [55]. Conditioned medium from isoproterenol-treated MSCs increased the expression of E- and P-selectin on bone marrow endothelial cells and cancer cell adhesion in an IL-1β-dependent manner. Together these findings delineated an important function for osteoblasts in promoting bone metastases that occurs in response to chronic stress and sympathetic activation.

An interesting novel role for osteoprogenitors in breast cancer bone metastases was recently demonstrated by Hao et al who investigated the osteogenic niche in an Osterix-Cre;$TDtomato^{f/f}$ and Osteocalcin-GFP mouse model in PyMT-N tumor bearing mice [56]. Osx-TD+ osteoprogenitor cells and osteocalcin-GFP+ osteoblasts significantly expanded at early stages of primary tumor growth (<0.2 cm^3) and even more once the tumor reached a volume of >1 cm^3. This finding was consistent with an increased $CD45^-Ter119\text{-}CD31^-CD51^+CD140a^+$ osteoprogenitor population in tumor bearing mice. Furthermore, a correlation between the increased osteogenic cell populations with a dislocation of hematopoietic stem and progenitor cells (HSPCs) that are replaced by an invading population $CD41^-$ granulocyte-monocyte progenitors (GMPs) resulting in aberrant myelopoiesis was found in PyMT-N tumor bearing mice. Using an osteoprogenitor ($Osx\text{-}Cre^+$) and osteoblast depletion ($Col1a1\text{-}Cre^+$) models, the authors showed that osteoprogenitors, but not mature osteoblasts are involved in the expansion of $CD41^-$GMPs in tumor bearing mice [56]. scRNA sequencing and GSEA analysis confirmed an enrichment in genes related to the expansion or proliferation of osteogenic cells in the respective clusters. NicheNetAnalysis revealed that osteoprogenitors highly express MMP-13 via secreted extracellular vesicles to disrupt the bone microenvironment and influence $CD41^-$GMP expansion. Osteoprogenitor depletion or MMP-13 inhibition sensitized the tumor cell response towards immunotherapies [56]. Kfoury et al. investigated the cell populations within the tumor stroma of human prostate cancer bone metastasis using scRNA sequencing. Interestingly, they found stromal cell populations inside the solid tumor microenvironment including osteoblasts, endothelium, pericytes and osteoclasts. These data indicate the importance of bone resident cells in tumor growth of bone metastasis [57].

4.4. Emerging techniques and novel targets in bone metastasis

Development of advanced techniques, including scRNA sequencing and bioinformatic tools are greatly expanding our knowledge on the bone metastatic environment. A bioinformatic approach was used to identify prognostic stemness-related signatures (PSRSs) and to study the "bone metastasis-specific regulation network" of invasive breast carcinomas [58]. The authors used clinical data and RNA sequencing data of human primary breast cancer samples with and without diagnosed bone metastasis from the Cancer Genome Atlas (TCGA) and identified differential expressed genes (DEG) using the edgeR method. mRNA stemness index (mRNAsi) was determined with regression, DEGs identified with weighted gene co-expression network analysis. It was proposed that CD248 (endosialin) is positively regulated by MAF protein resulting in the upregulation of the apical junction pathway as the bone metastasis-specific regulation network as trifluoperazine as the possible inhibitor of this network identified using Connectivity Map [58]. These findings of the regulation network were confirmed with spatial scRNA sequencing, ChIP-Seq and multi-omics. CD248 is expressed by mesenchymal stem cells and osteoblasts besides other cells and has been shown to have a function in tumor invasion, ECM adhesion and activation of MMP9 in tumor metastasis (Fig. 2B). Noteworthy, MORAb-004/ontuxizumab, a human monoclonal antibody that targets CD248, is evaluated in pre-clinical trials in cancer [59].

Together, these data show that osteogenic cells are important in the maintenance and activation of cancer cell colonization, quiescence and proliferation (Fig. 2). In addition, osteolineage cells modulate immune cell response and chemotherapy sensitivity. Current progress in the knowledge of metastatic skeletal disease emphasizes the role of osteoprogenitors, pre-osteoblasts and osteoblasts, as well as the bone microenvironment in early steps of bone metastasis colonization (Table 1) and therefore should be further considered as a target to combat bone metastatic growth.

5. Osteoblasts as cellular targets in bone metastases

Given the osteolytic nature of breast cancer bone metastases, the current bone targeted therapies are antiresorptive drugs bisphosphonates and Denosumab. Similarly, since prostate cancer-induced osteoblastic bone metastases are associated with high bone turnover and thus high resorption, antiresorptive therapies are effective in delaying and preventing SREs in prostate cancer bone metastases [60]. Although bisphosphonates and denosumab prevent further SREs and alleviate pain, they do not prolong survival and do not heal the existing lesions [61]. This raises the question whether increasing osteoblast function and bone formation could be an approach for future treatment modalities. Currently, three bone anabolic agents are approved for the treatment of severe osteoporosis [62]. Teriparatide (PTH 1–34) and Abaloparatide (a synthetic analog of PTHrP (1–34)) activate the PTH receptor and downstream signaling while Romosozumab, an antibody targeting the Wnt inhibitor sclerostin activates the Wnt signaling.

5.1. PTH as a regulator of cancer colonization and growth

Intermittent PTH increases bone mass through a remodeling-based mechanism by increasing the number and activity of osteoblasts and bone formation, and subsequently the number activity of osteoclasts and bone resorption. In prostate cancer, PTH-induced expansion of the osteoblast-lineage cells has been shown to increase the number of metastatic prostate cancer cells in the bone [63]. In breast cancer, preclinical studies showed that intermittent anabolic (4 weeks) PTH treatment decreases the incidence of spontaneous metastasis to bone in syngeneic and immunocompromised orthotopic breast cancer model. Furthermore, anabolic PTH treatment reduced cancer cell engraftment, alleviated tumor growth in bone and protected bone integrity in an intratibial model of osteolytic disease. Finally, PTH treatment prolonged survival of cancer-bearing mice [64]. Since no difference in metastases was observed in visceral organs, the prolonged survival is likely a consequence of reduced bone metastases. Mechanistically, intermittent PTH impaired the osteoblast-induced cancer cell migration by reducing the expression of several pro-migratory cytokines, including Vcam-1, RANKL, CXCL-12 and several MMPs. Inhibition of Vcam-1 with a neutralizing antibody reduced cancer cell migration towards osteoblasts in vitro and decreased metastases to hind limbs in vivo. Together the results suggest that PTH inhibits breast cancer cell homing to bone by reducing the expression of cytokines that are important for the preparation of pre-metastatic niche (e.g. RANKL and MMPs) and cancer cell recruitment (e.g. Vcam1 and CXCL-12). The effect of PTH in reducing bone metastases is mediated by the PTH1R in both osteoblasts and breast cancer cells [64,65]. This is evidenced by an impaired ability of PTH to reduce bone metastases in mice with ablated PTH1R signaling in osteoblasts or when PTH1R was deleted in 4T1 breast cancer cells. Interestingly, in breast cancer cells PTH decreased the expression of PTHrP, one of the drivers of the vicious cycle [65]. These findings thus propose a new mechanism through the PTH-PTH1R-PTHrP-interplay that might contribute to bone metastases.

In contrast to anabolic (4 week) pre-treatment or treatment, an intermittent short-term administration of PTH (daily for 5 days) prior to intracardial injection of MDA-MB-231 breast cancer cells had no effect on cancer cell homing to hind limbs [66]. Instead, tumor burden in skeletal sites outside the hind limbs (e.g. ribs and tail) was increased in this setting. These results suggest that while PTH has no effect on cancer cell homing to hind limbs, it makes the other skeletal sites more permissive for cancer cell homing possibly by altering the bone

Table1
Identified osteolineage cell subpopulations that contribute to the regulation of the early metastatic niche in bone metastasis.

Osteolineage cell type	Marker expression	Observed effect	Reference
"educated" osteoblasts	Runx2, osteocalcin, osteopontin, increased expression of Col1a1, MMP3	Support dormancy	[35]
"uneducated" osteoblasts	Increased IL6, αSMA, VEGF, no difference in ALP, Col1a1, MMP3	Unknown	[37,38]
SNO-cells (pre-osteoblasts)	N-cadherin+/CD45- osteoblasts	maintain quiescence via Notch/Jagged1, enrichment of dormant DTC in SNO niches	[37,38]
ALP+, Col1+ osteogenic cells	Osx+, Runx2+, β-catenin, LEF1	DTC localize in ALP+ Col1+ osteogenic cell niche, active osteogenesis	[29]
ALP+ osteoblasts	ALP+	Regulated by tumor derived SCUBE2, SCUBE2 KD depleted number of ALP+ cells, downregulation of osteolineage genes and pathways contributing to ossification and collagen formation;upregulation of mTOR signaling via heterotypic adherens junctions	[46]
Osx-TD+ osteoprogenitors	CD45-Ter119-CD31-CD51+ CD140a+ osteoprogenitor	Expanded at early stages of primary tumor growth, resulted in dislocation of HSC and expansion of CD41-GMPs and aberrant myelopoesis by increases sEV MMP-13 secretion	[56]
OCN-GFP+ osteoprogenitors		Expanded at early stages of primary tumor growth	[56]

microenvironment, particularly the osteoblasts. The difference between the study outcomes can likely be explained by distinct experimental conditions used in the studies. However, the findings consistently suggest that modifying the osteoblast lineage cells and their activity by PTH receptor influences breast cancer cell engraftment and growth in bone.

5.2. Targeting sclerostin in osteolytic bone metastases

Activation of Wnt signaling by sclerostin antibody (Scl-Ab) increases bone formation while reducing bone resorption leading to an increased bone mineral density and reduction of fracture rate in post-menopausal women with severe osteoporosis [67]. However, Wnt signaling is also implicated in tumor progression and metastasis in various cancers, including breast, raising some concerns of using Scl-Ab in cancer indications [68]. Pre-clinical studies of breast cancer bone metastases have shown that Scl-Ab treatment of established metastases reduces tumor growth in bone without affecting metastasis to other organs in a model of intracardiac injection of MDA-MB-231 cells [69]. Furthermore, Scl-Ab protected bone from cancer induced destruction upon intracardiac or intratibial injection on breast cancer cells [69,70]. Similar effects were seen when sclerostin was inhibited using a small molecule inhibitor [71]. Like in osteoporosis models, the strong bone protection is mediated by a dual effect of increased bone formation and reduced bone resorption [69,71]. Furthermore, inhibition of sclerostin either by Scl-Ab or by small molecule prolonged survival of cancer-bearing mice. Interestingly, sclerostin is expressed by bone metastatic breast cancer cells [69–71] and inhibition of breast cancer-derived sclerostin in SPC2 cells reduced cancer cell growth in bone, suggesting that a systemic inhibition might act through the bone microenvironment and the cancer cells [71]. Although the focus of this review is bone metastases, it is worth to mention that Scl-Ab treatment also protected bone from multiple myeloma (MM)-induced bone destruction in two independent pre-clinical studies [72,73]. In MM no effect on cancer progression or bone resorption was observed, suggesting that the osteoblasts are the main cellular target in this disease condition.

Similar to PTH, timing of Scl-Ab administration seems to affect disease outcome. In a study in which Scl-Ab was administered one week before breast cancer cell inoculation, Scl-Ab increased tumor growth in bone [74]. However, this effect was only observed in an immunocompromised model with MDA-MB-231 cells while no difference between control and Scl-Ab was seen in immunocompetent models using 4T1 or E0771 breast cancer cells. Pre-treatment with Scl-Ab did not affect orthotopic tumor growth in any of the models. A possible explanation for the increased tumor burden of MDA-MB-231 cells is their higher responsiveness to Wnt ligands compared to other 4T1 and E0771 cells [74].

5.3. New approaches to target the osteoblast lineage cells

In addition to the more established targets, new pharmacological approaches to target the osteoblast lineage cells are being discovered. The TAM family of receptor tyrosine kinases consisting of TYRO3, AXL and MERTK and their cognate ligands Gas6 and protein S (PROS1) induce phosphorylation and activation of multiple downstream signaling pathways. TAM receptors and their ligands are frequently overexpressed in cancer and mediate tumor stroma interactions. In MM, Axl was shown be highly expressed in dormant tumor cells that reside in the endothelial niche. Together with other dormancy genes high Axl expression predicted increased survival in patients [75]. Consistently, targeting Axl using small molecule inhibitors such as cabozantinib and BMS-777607 released MM cells from a dormant state and promoted their proliferation [75].

Recently, TAM receptors MERKT and TYRO3 were shown to regulate osteoblast differentiation and bone formation [76]. While osteoblast-targeted deletion of TYRO3 reduced bone formation and bone mass, deletion of MERTK increased osteoblast number and bone formation resulting in an increased bone mass. Consistently, pharmacological inhibition of MERTK using an orally bioavailable, potent, and selective small molecule inhibitor R992 increased osteoblast differentiation in vitro and bone formation in vivo. Furthermore, R992 prevented breast cancer-induced bone loss upon intracardiac injection of MDA-MB-231 breast cancer cells. Consistent with the observations in healthy mice, R992 increased P1NP and osteoblast number while osteoclast parameters were unchanged [76]. Although more investigation is needed, these studies suggest that targeting the osteoblast lineage cells by bone anabolic treatments could be a future approach to treat osteolytic bone metastases.

6. Conclusions and future perspectives

The crucial role of the vicious cycle and particularly the osteoclasts in promoting cancer cell growth is established. During the recent years, it has become evident that osteoblasts are not only inactive bystanders providing RANKL and thus indirectly supporting the vicious cycle of bone metastases but have an active role in regulating the fate of cancer cells. Through various mechanisms osteoblast lineage cells mediate cancer cell homing to bone, support DTC dormancy and contribute to metastatic relapse. Although several reports indicate that osteoblasts and their precursors promote early stages of metastases, their function is suppressed during the progression of osteolytic disease. Thus, increasing osteoblast function provides an attractive therapeutic option to treat osteolytic metastases. In support of the concept, several pre-clinical studies have demonstrated beneficial effects of bone anabolic drugs in breast cancer bone metastases. Despite the promising pre-clinical evidence, clinical studies are pending, and no anabolic agents are approved to be used in the clinics. Therefore, more pre-clinical and clinical studies are urgently needed to resolve some inconsistencies between current animal studies.

Until now, most reports have considered osteoblasts as a rather homogenous cell population and studied their role as such. However, during the recent years, osteoblasts and their precursors have been shown to represent heterogenous cell types with distinct locations and functions [7,12]. Interestingly, an SSC population was very recently identified that is specifically located in the spine and gives rise to vertebral osteoblasts [77]. These vertebral SSCs express transcription factors ZIC1 and PAX1 and secrete high levels of MFGE8. Intriguingly, the authors showed that SSC-derived MFGE8 recruits metastatic cancer cell to the vertebrae, explaining at least in part the trophism of metastatic cancer cells to the spine. Although several questions on the specific function of these SSCs in bone metastases remain open, the findings open an interesting new direction on the role of specific osteoblast/SSC populations in bone metastases [77]. Future studies will reveal whether certain subpopulations specifically support homing, dormancy and/or relapse, which could explain the multiple and partially conflicting functions of osteoblasts in dormancy and disease progression. In depth understanding of osteoblast (sub)populations could possibly also reveal novel targets to combat the incurable disease.

CRediT authorship contribution statement

Jennifer Zarrer: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Hanna Taipaleenmäki:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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